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An-Najah National University

Faculty of Graduate Studies

Relationship between Factor V Leiden Mutation and Poor Pregnancy Outcomes

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master in Public Health, Faculty of Graduate Studies, An-Najah National University, Nablus, Palestine

2007

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This thesis was defended successfully on 15 / 12 / 2007 and approved by

<u>Committee members</u> **<u>signature</u>**

1- **Dr. Ayman Hussein (Supervisor)---**
2- **Dr. Hesham Darwish (Internal examiner)-------------------------------**
3- **Dr.Ala`a Salah (External examiner)--------------------------------------**

Dedication

To my parents, brothers, sisters. To my wife, sons, and daughters.

Acknowledgement

I am sincerely grateful to my thesis supervisor Dr. Ayman Hussein for his continuous encouragement and guidance. During my time in his laboratory, I have had the due independence and flexibility, which have paved the way to acquire more experience and to highly appreciate my research. My ability to research has been developed under his direction and I will always be grateful for the opportunities he has given me.

I would also like to express my thanks to Dr. Hisham Darweesh and Dr. Aidah Al-Kaisi for their fruitful advice and help during the period of my work.

I wholeheartedly extend my thanks to the following persons for allocating me enough time out of their busy schedules to help and to support me; thanks to Dr. Ibrahim Sulqan, Dr. Umayya Khammash, Dr. Moh. Khalili, Dr. Husam Siam, Dr. May Keileh, Dr. Ala`a Salah.

From the bottom of my heart, I would also like to extend my sincere to all health staff in the UNRWA health division, especially to those in the

health centers in Nablus Area for their help and assistance; I will always treasure the friendships and the moments that we have shared together.

I would like to thank my family for the support and the encouragement they expressed during the period of my study.

I also extend my thanks to UNRWA Health Division and Faculty of graduate studies for their financial support.

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Abstract

Pregnancy is a hypercoagulable state, with increased tendency for thrombus formation. That is increased if pregnancy is combined with thrombophilia. Thrombophilia could be acquired or inherited. Among the inherited types is Factor V Leiden mutation, an autosomal dominant disorder. The mutation is believed to be a major inherited risk factor for venous thrombosis. Recently, it was suggested that this mutation, through stimulation of venous and placental thrombosis events, were strongly associated with different pregnancy adverse outcomes, including PET, recurrent miscarriages, IUGR, IUFD, abruption placenta, and others. Although other studies disputed such a link. The aim of our study was to investigate the relationship between Factor V Leiden mutation and some

adverse pregnancy outcomes, namely recurrent miscarriages and PIH. In this case-control study where 137 participants with adverse pregnancy outcomes (*66 First trimester RM, 25 second trimester RM, 26 with IUFD & 20 with PIH*) were compared to 155 women with uncomplicated pregnancies. Blood samples were collected from participants for DNA extraction; and Factor V Leiden mutation was identified using PCR The mutation was confirmed in 35 cases out of 137 (25.5%), and in 13 out of 155 controls (8.4%). .The relationship between the mutation and recurrent miscarriages was established using SPSS analysis version 15 [*Odds ratio was 3.75 p-value=0.000*]

Comparing those with ***first trimester abortion cases*** and the ***control*** group *odds ratio was 2.45 p-value=0.029*, while the ratio was 10.5 and *p value* was 0.00 when comparing those with ***second trimester abortions cases*** to the ***control,*** a comparison between women with ***IUFD*** and the ***control*** group in relative to the Factor V Leiden mutation, the *odds ratio was 4.5 and p-value was 0.000. Finally* comparing women with ***PIH*** to ***controls***, in relative to the prevalence of Leiden mutation no significant difference existed between the two groups

These results suggest a strong correlation between recurrent miscarriages and Factor V mutation in our population.

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• **APC**	*Activated Protein C*
• **ARMS**	*Amplification refractory mutations system*
• **DVT**	*Deep Vein Thrombosis*
• **IUFD**	*Intra Uterine Fetal Death*
• **PCR**	*Polymerase chain reaction*
• **PET**	*Pre-Eclampsia*
• **PIH**	*Pregnancy Induced Hypertension*
• **PPO**	*Poor Pregnancy Outcomes*
• **RM**	*Recurrent Miscarriages*
• **RT**	*Room Temperature*

Chapter One

Introduction

Introduction

Pregnancy is a valuable process in our life. It is the natural goal for each family to get their child. The process involves carrying a fetus in the uterus of the woman as a product of conception and fertilization. However, pregnancy is a complicated physiological process that might lead to negative outcomes and could threaten the women`s life or the fetus. .

Physiological changes during pregnancy

Different physiological changes occur during the process of pregnancy, which affect all of the woman systems; these include metabolic adaptations and hormonal changes. However, we are interested in those changes that affect the coagulation factors. It is due to secondary increase in the concentrations of pre-coagulant factor , a reduction of the naturally occurring anticoagulant proteins and increase in fibrinogen ,that characterized pregnancy with hypercoagulability [1,2,3,4] **.** A successful pregnancy is highly dependent on the establishment and maintenance of an adequate placental circulation [5]. It has been postulated that the abnormalities of placental vasculature leading to inadequate fetomaternal circulation are responsible for some poor pregnancy outcomes [6, 7].

Different poor pregnancy outcomes could accompany the pregnant woman including abortion (either during the first or second trimester), intra uterine fetal death (IUFD), intra uterine growth restriction (IUGR), still birth (SB), preeclampsia (PET), abruption placenta [6, 7]. More specifically recurrent miscarriages (RM) are a frequent health problem where the worldwide incidence of RM is about 0, 5-1 % of pregnant women [6, 7].In Palestine, the incidence of abortion is 4-8% of Palestinian pregnant women attending UNRWA antenatal care in the West- Bank [8]. It should be noted that both inherited and acquired factors may play a role as a cause of abortion; risk factors include parental chromosomal abnormalities (2%-3%), uterine abnormalities (10%-15%), hormonal imbalance (25%) [6].The cause of RM could not be established in approximately 50% of cases [6]. Changes in blood coagulation may play a role in abortion occurrence, since haemostatic disorders may cause an obstruction of the placental vessels [3]. These physiological changes in the coagulation system may increase the risk of pregnancy failure if are associated with thrombophilia.

Thrombophilia

Thrombophilia is either inherited or acquired conditions that predispose the affected person to an increase risk of thromboembolism. The relationship between recurrent pregnancy loss and some types of thrombophilias included abnormalities in protein C, protein S and antithrombin deficiencies has been well studied[7] .There are three important inherited thrombophilias which are responsible for the majority of thromboembolic cases among patients with otherwise no apparent other risk for thrombosis[7] . One of these later discovered thrombophilias is Factor V Leiden mutation [7] .

Factor V Leiden mutation

Is an autosomal dominant disorder [9] characterized by an impaired anticoagulant response to activated protein C (APC) [9]. During hemostasis, APC inactivates factors Va and VIIIa and thereby limiting clot formation. Inactivation of factor Va normally occurs through cleavage at the arginine 506[10]. Mutant factor V molecules that have glutamine instead of arginine at position 506 (R506N)[10] in the primary structure of the protein become

procoagulant rather than anticoagulant [10] (See figure 1). Resistance to the anticoagulant activity of APC has been described as a major cause of predisposition to thromboembolism [11]. APC resistance accounts for about 21%of deep vein thrombosis (DVT) in people younger than 70 years and up to 50% of familial venous thrombosis [10, 12]

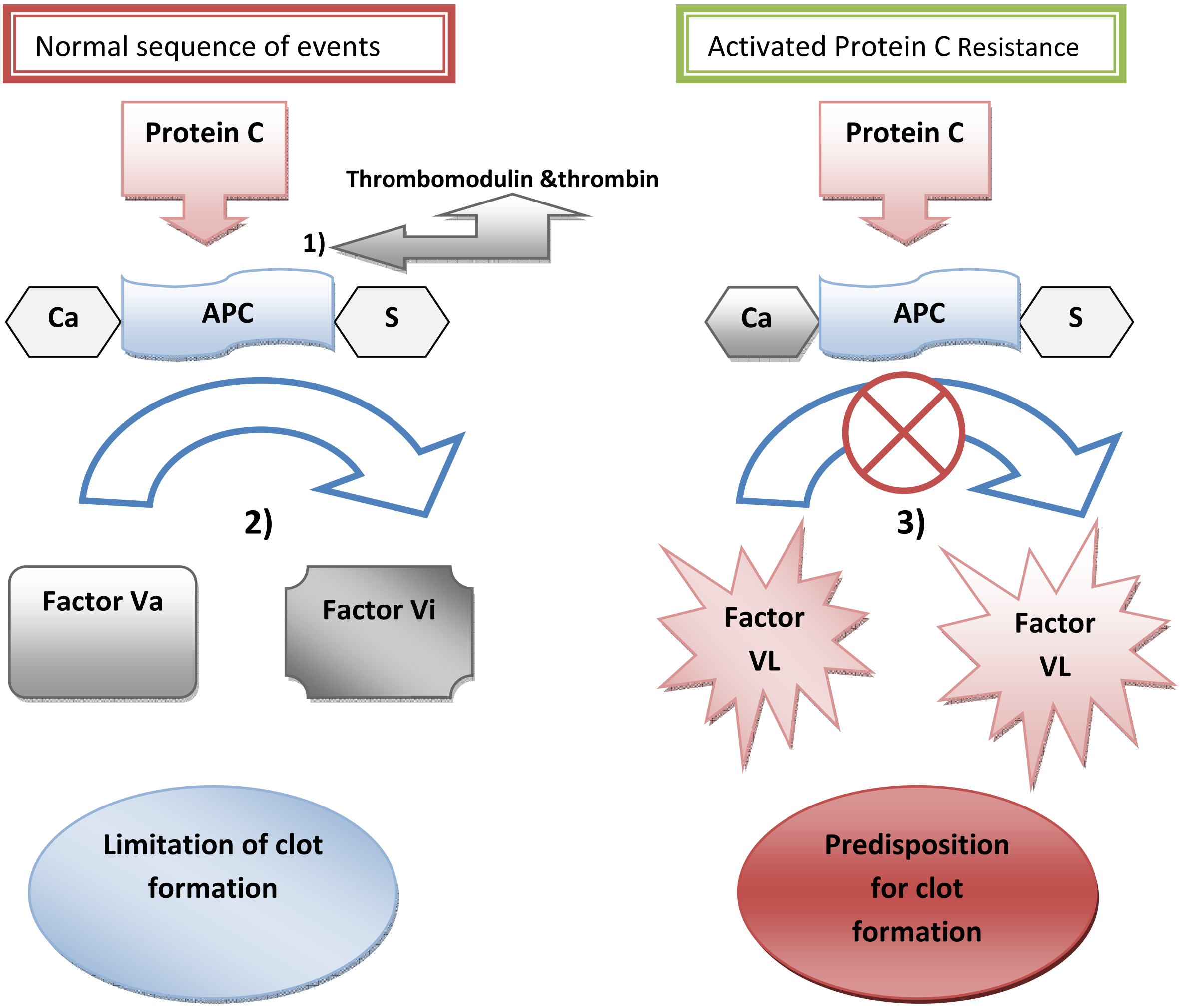


Fig (1): Activated protein C anticoagulant pathway

1) *Thrombin binds to an endothelial cell receptor, thrombomodulin, and becomes an anticoagulant protein capable of activating protein C.*
2) *In the presence of protein S and Ca , activated protein C inactivates coagulation factors Va and VIIIa. This is the mechanism whereby clot formation is limited.*
3) *Factor V Leiden is a mutation in the factor V molecule, rendering its resistant to cleavage by activated protein C. Factor V remains a procoagulant and thus predisposes the carrier to clot formation.*

Factor Va- active factor V **Factor VL**- Leiden Factor V
Factor Vi inactivated Factor V

The mutant Factor V Leiden protein is inactivated at a 10-fold slower rate than normal protein, and persist longer leading to the generation of a prothrombotic state [13]

Literature review

Prevalence of Factor V Leiden Mutation in Different Population

Factor V Leiden mutation is prevalent among the Caucasian people, with 4.4%-10% incidence rate. Heterozygous genotype is found in 5-8% of Caucasian population and is associated with 4-8% fold increased of the relative thromboembolic risk [10, 14]. The homozygous genotype is found in 1of 5000 person, it is associated with 80 fold increase of thrombotic risk, and it contributes to a large proportion of the population–attributable risk of venous thromboembolism [10, 14].

Several studies on the relationship between factor V Leiden mutation and poor pregnancy outcomes were reported. Kupfermen et. al.[15] conducted a case control study in order to estimate the prevalence of Factor V Leiden mutation among women with at least one poor pregnancy outcome, Factor V

Leiden mutation was confirmed in significantly higher prevalence among cases than that among controls. Their study concluded that pregnant women with serious complications have an increased incidence of factor V Leiden mutation, predisposing them to a higher risk of developing thrombosis compared to the control group [15]. Another case control study conducted by Finan et al. [16] with an objective to determine the prevalence of factor V Leiden mutation and other thrombophilic mutations among Lebanese women with recurrent idiopathic abortions. In this study Leiden mutation was found among 41% of cases and among 16% of the control group. About 15.6 % of the carrier cases were homozygous for the mutation and 84.4% were heterozygous, however all carriers among the control group were heterozygous [16]. In conclusion, they came up with that Leiden mutation is a major inherited risk factor associated with primary habitual abortions [16]. Similar association was reported by Mahjoub et. al. [17] who conducted another case control study aiming to determine the prevalence of Leiden mutation among Tunisian women with 3 or more consecutive early, late and early and late recurrent abortions. The authors compared cases to control

women with no history of complicated pregnancy. The results showed 27% prevalence of Leiden mutation in the case group compared to 11.5% in the control group[17], the conclusion was that Leiden mutation is a risk factor for early and late pregnancy loss The relationship between Leiden mutation and unexplained late fetal loss was established by Martinelli et al. [18] in a case control study. Two groups of Italian women were compared for the prevalence of Leiden mutation among them. The first group consisted of women with unexplained late fetal loss, while the second group consisted of control women with uncomplicated pregnancy. Unexplained late fetal loss was linked to the presence of Factor V Leiden mutation [18]. The relative risk of 3.2 was calculated for women with late fetal loss compared to women with uncomplicated pregnancies [18]. Foka et al. [19] suggested the role of Factor V Leiden mutation as an additional risk factor for recurrent miscarriages. In this report, 19% of Greek women with recurrent miscarriages have the mutation compared to 4% of women with uncomplicated pregnancy [19]. Similar results were also reported by Grandone et al. [20] who conducted a case control study in Southern Italy, in this study 16.3% of women with recurrent

miscarriages have the mutation compared to 4.2% in the control group [20]. The authors concluded that the prevalence of factor V Leiden mutation is significantly higher among cases and the mutation was more particularly associated with late recurrent miscarriages [20]. Such association was not established by Hashimoto et al [21] , in a case control study among Japanese women, where the results showed no significant difference in the prevalence of Factor V Leiden mutation among the two groups[21] these authors concluded that Factor V Leiden mutation is not associated with recurrent pregnancy loss in Japanese population[21]

The association of Factor V mutation with recurrent abortions was studied among Israeli women by Younis et al. [22] and showed that 16% of women with recurrent first trimester abortions have the mutation compared to 22% of women who had abortion in their second trimester [22]. The prevalence of Factor V mutation was found to be 6% among women with uncomplicated pregnancies [22]. The authors concluded that factor V Leiden mutation seems to be linked to first and clearly more linked to second

trimester recurrent pregnancy losses [22] .Whereas Balasch et al. [23] did not found such association in a case control study included Spanish women with recurrent first trimester abortion and women with uncomplicated pregnancies, where the mutation was found in only one case and one control. Another investigation on the role of Factor V mutation with unexplained recurrent abortions had been done by Brenner et al. [24] They held a case control study, which included the case group of Israeli women with unexplained recurrent abortions; the control group included women with history of uncomplicated pregnancy, they studied the two groups for three thrombophilic mutations, including factor V Leiden mutation. The result showed that 32% of cases had the mutation compared to7% of controls [24]. The authors concluded that there was an association between factor V Leiden mutation and recurrent pregnancy loss [24]. The association between Factor V Leiden mutation and unexplained recurrent miscarriages was established in another case control study conducted by Souza et al. [25] among Brazilian women with three or more fetal losses for the Leiden mutation, and compared them to women with successful pregnancies. The mutation was

found in 7.1% of cases and in 1.6% controls [25]. They again concluded that an association between factor V Leiden mutation and recurrent fetal loss was noted [25]. In contrast Preston et al. [26] failed to establish a significant association between Leiden mutation and recurrent pregnancy losses in European women participating in the European Prospective Cohort on Thrombophilia. The same result as Preston was achieved by Dizon – Townson et al. [27] who studied 40 American women with recurrent fetal loss and compared them to 25 women control group. The mutation was not detected in either group.

Chapter Two

Methodology

Methods

Case control study was conducted to study the relationship between factor V Leiden mutation and poor pregnancy outcome (PPO) among Palestinian women in the North West-Bank. The study was approved by the Institutional Review Board at An-Najah National University in Nablus.

Poor pregnancy outcomes, that we are interested in, are: recurrent fetal losses in the three trimesters and pregnancy induced hypertension (PIH).

The definitions of the above mentioned outcomes were taken from the National Unified Reproductive Health Guidelines & Protocols published by the Palestinian Ministry of Health in 2000.

Pregnancy losses were classified into three categories:

1. **First trimester abortion**: the pregnancy loss within the first 12 gestational weeks.
2. **Second trimester abortions**: the pregnancy loss between the 12-th and the 22nd gestational week.
3. **Third trimester pregnancy loss**: fetal loss after the 22nd gestational week and is considered as Intra Uterine Fetal Death **(IUFD)**.

Pregnancy Induced Hypertension (PIH): increased blood pressure after 20 gestational weeks of ≥140\90mm Hg.

Women experienced one or more recurrent pregnancy loss and/or PIH, were eligible to be included as potential cases. Study cases were identified retrospectively by reviewing their antenatal records at UNRWA health centers using the following criteria:

- Three or more first trimester abortions
- Two or more second trimester abortions
- One or more third trimester pregnancy loss (IUFD)
- Pregnancy Induced Hypertension.

Samples discription

Participants were divided into two groups: case and control group.

Cases

137 women who experienced one or more of the adverse pregnancy outcomes mentioned above during their reproductive age were identified by reviewing the antenatal records in the health centers of UNRWA health department in North West Bank.

Inclusion Criteria

Every woman who has at least one of the above fore mentioned outcomes was invited to participate in the study as a case.

Controls

Comprised of women with at least 2 normal pregnancies and without any history of adverse pregnancy outcome or recurrent miscarriages.

Exclusion criteria

Woman who has any of the following criteria was excluded from the study groups:

- A history of vascular thrombotic disease,
- Pre-existing diabetes mellitus,
- Pre-existing renal disease,
- Fetal congenital anomalies,
- Fetal chromosomal anomalies,
- Uterine abnormalities,
- Multiple pregnancy,
- Pre-existing hypertension,
- IUFD with known cause,

- Women known to have the mutation
- A known causes of the abortion.

Tools

A uniform questionnaire was used to collect information about age, parity, medical and obstetric history, smoking, family medical and obstetric history, residency, refugee status, relative marriage, and the educational level. The questionnaire was developed by the author. A pilot study was conducted on 27 persons including: 2 medical doctors, 1 dentist, 2 staff nurses, 2 midwives, 5 practical nurses, 2 cleaners, 10 patients, 3 teachers.

The data was collected by a direct interview between the researcher and each participant.

Blood collection for DNA extraction

Five ml venous blood was collected from each participant into EDTA tubes after consent obtained from each participant.

DNA was extracted from the blood samples using Master pure DNA purification kit for blood (cat. No. MG71100, Epicenter Biotechnologies, Wisconsin, USA). The procedure is based on using Buffy coat, according to the following steps:

- Blood collected from cases and controls were kept at 4 C^0 for 12-18 hours.
- Buffy coat was separated by centrifugation at 1000g x 15 min.
- 300 µl Buffy coat were collected and mixed with 1200 µl Lyses Buffer1 in 1.5ml eppendorf tubes. Samples were mixed by inversion and incubated at room temperature (RT) for 5 min., sample mixing was repeated and the incubation was extended for 5 additional minutes at RT.
- A final inversion mixing was performed at the end of the incubation time then the samples were centrifuged at 10.000xg for 25 sec.
- After centrifuging the supernatant was removed by aspiration keeping around 25 µl with the red pellet. The pellet was vortexed for uniform suspension.
- 600 µl Lyses Buffer 2 were added to each sample and mixed by pipetting up and down 7-10 times to lyse the cells
- 300 µl of Precipitation Solution were added to the mixture, followed by continued vortexing for 30 seconds.
- Samples were then centrifuged at 10.000xg for 10 min. The white supernatant moved into a new clear tube.

- DNA was precipitated by adding 500 µl cold isopropyl alcohol followed by centrifugation at 10.000xg for 10 min.
- The supernatant was removed and the formed pellet was washed using 200 µl of cold 70% ethanol.
- After centrifugation at 10.000xg for 10 minutes pellet was dried and dissolved using 50 µl TE Buffer.
- DNA was used immediately for PCR analysis or stored at -20 C^0 until used.

Agarose Gel Electrophoresis

The DNA products were separated on a 4-mm thick horizontal 1.5% agarose (Sigma Ltd.) gels prepared in EDTA (TAE) buffer, with the addition of 0.5 µg Ethidium Bromide/ml. 1 µl of loading buffer was added to 2 µl DNA. Each gel was allowed to run in TAE buffer for 15-20min. at 100 volts and later visualized under UV light (figure 2).

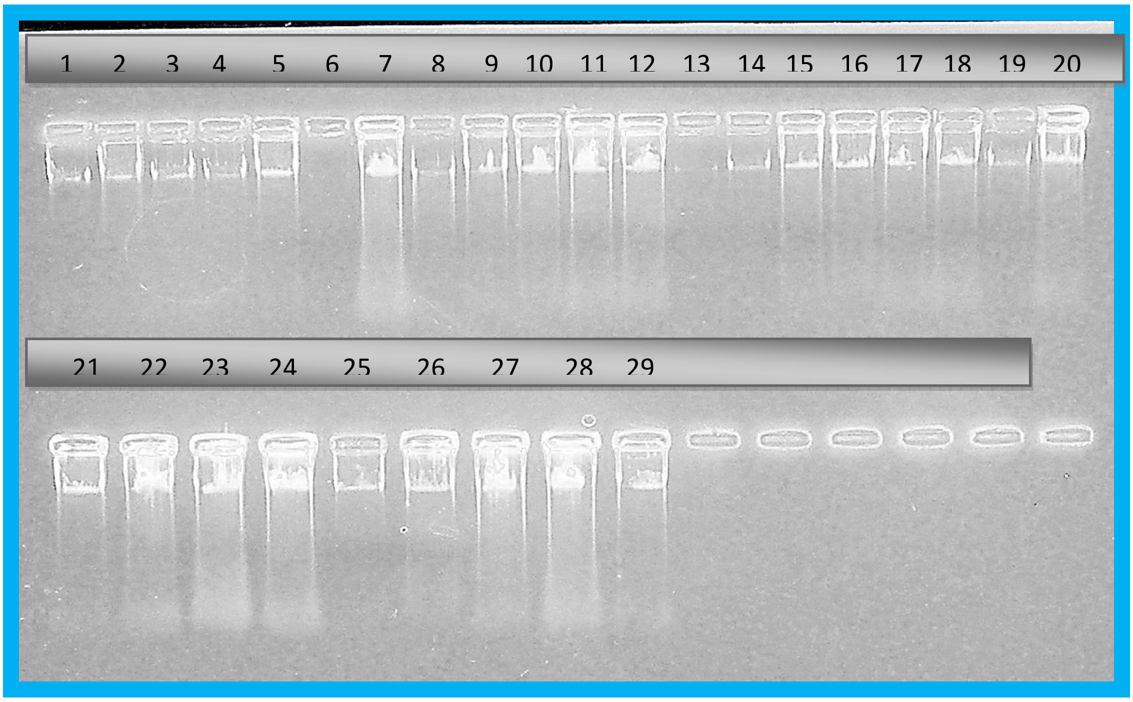


Fig-(2) 1.5 Agarose gel electrophoresis showing resolved genomic DNA isolated from human WBCs.

Identification of Factor V mutation by Polymerase Chain Reaction (PCR)

In order to identify Factor V Leiden mutation, the polymerase chain reaction (PCR) method was utilized combined with the amplification refractory mutation system (ARMS) using Minicycler.

ARMS is an amplification strategy in which a polymerase chain reaction primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue. Thus, amplifying

a specific member of a multi-allelic system while remaining refractory to amplification of another allele that may differ by a single base from the former. Hence, the presence of an amplified product indicates the presence of a particular allele and vise versa. ARMS is based on the principle Thermus aquaticus (Taq) polymerase, the DNA polymerase commonly used in PCR, lacks a 3` to 5` exonuclease activity and thus a mismatch between the 3` end of the PCR primers and the template will result in greatly reduced amplification efficiency. The principles of this method are illustrated bellow *in figure 3*. Three primers are utilized in ARMS: Common primer that is chosen to flank the site of the suspected mutation, Normal primer, in which the last nucleotide at the 3` end is chosen to match the corresponding nucleotide in the normal sequence and Mutant primer, in which the last nucleotide at the 3` end is chosen to match the mutation point and to mismatch the normal sequence under appropriate specific conditions. The main advantage of ARMS is that the amplification step and the diagnostic steps are combined, in addition to being an accurate, rapid and a simple method

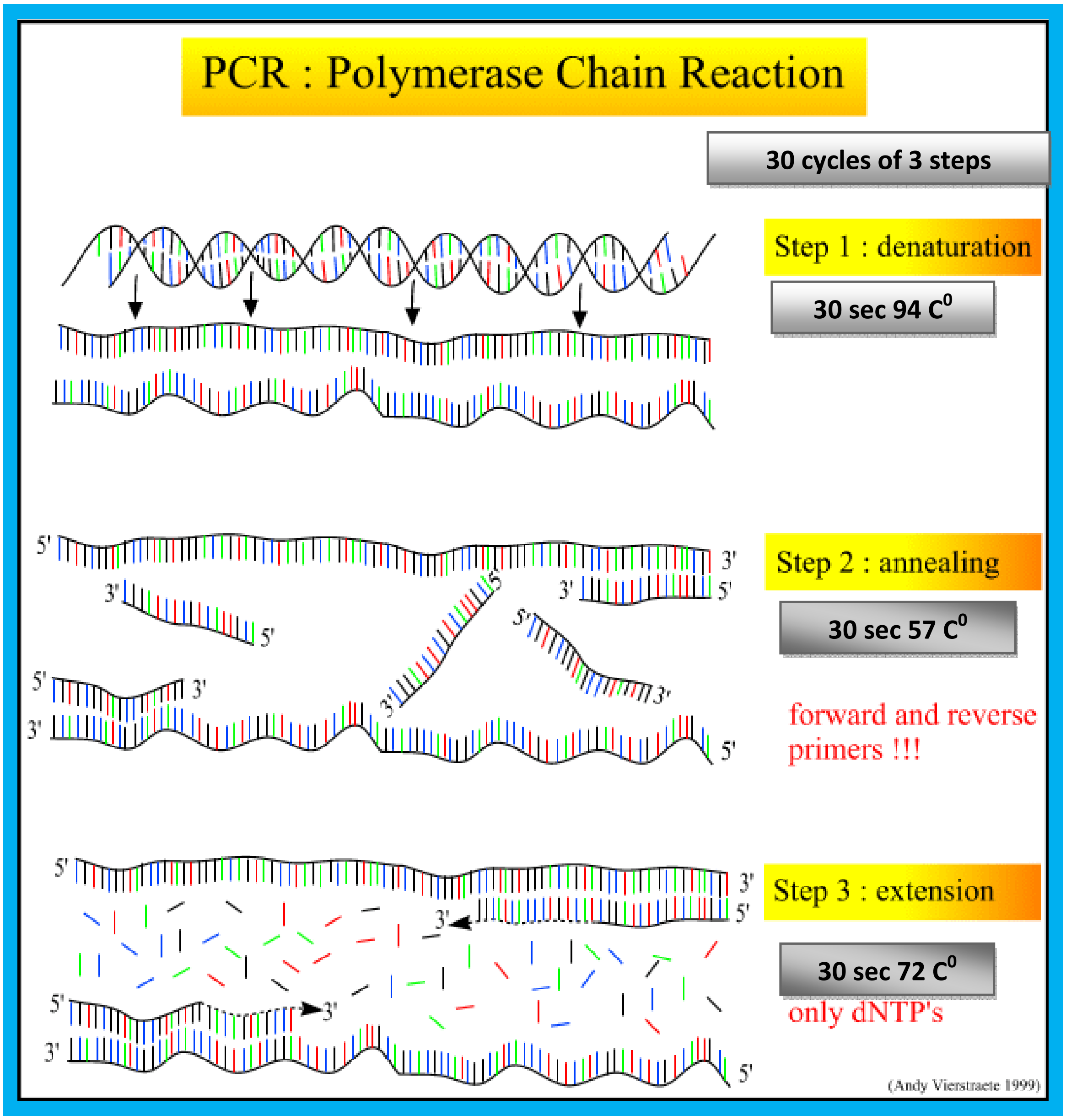


Fig-(3) PCR principles

Adopted with modification from
http://www.flutrackers.com/forum/showthread.php?t=11286

Primers sequencing

- Control primer (C):
  - 5` *CGCAGGAACAACACCATGAT* **3`**
- Normal primer (N):
  - **5`** *AACAAGGACAAAATACCTGTATTCATC* **3`**
- Mutant primer (M):
  - 5`*GTCTGTCTGTCTCTTCAAGGACAAAATACCTGTATTCTTT* **3`**

Two PCR reaction mixture were prepared , one with Normal primer and the other with Mutant primer , each mixture was of **30 µl** volume containing:**1 µl** (0.2 µg) of *either primer*(N or M-primer) , **1 µl** (0.2 µg) *C-Primer*, **2.5. µl** *(10X) Buffer*, **2.0 µl** *dNTP* mixture (2.5mM each), 0.125 **µl** (0.625U) *Enzyme*, **2 µl** (1-1.5µg*) DNA* and up to **30 µl** sterile water

The amplification program of the PCR was performed as follows:

- ***Denaturation*** at **94 C^0** for 30 sec.
- ***Annealing*** at **57 C^0** for 30 sec.
- ***Extension*** at **72 C^0** for 30 sec
- ***Number of cycles*** was ***30***
- ***Final extension*** at 72 **C^0** for 5 min.

Agarose Gel Electrophoresis for PCR products

The PCR products were separated on a 4-mm thick horizontal 1.5% agarose gel,(Sigma Ltd.) prepared in EDTA(TAE) buffer, with the addition of 0.5 µg ethidium bromide/ml. 2 µl of loading buffer was added to 10 µl DNA Each gel was allowed to run in TAE buffer for 1hour- , at 100 volts and later visualized under UV light (figure 3 & 4)

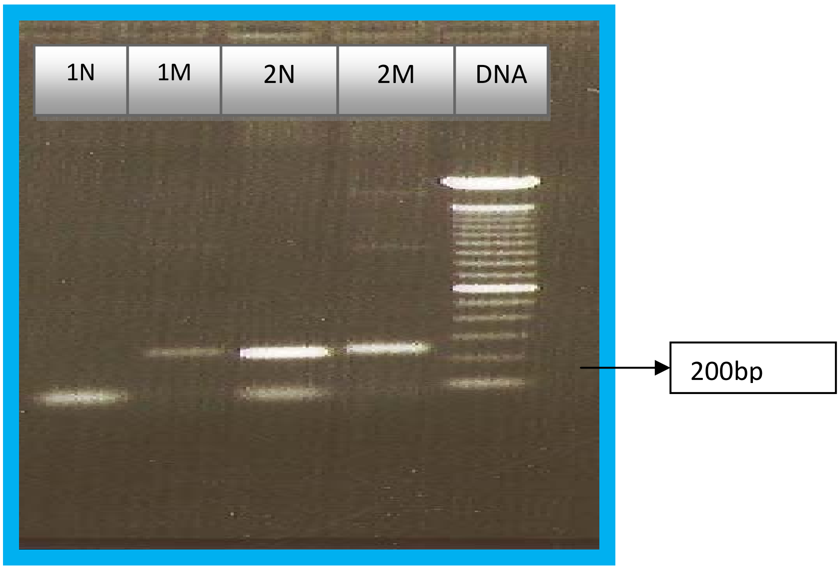


Fig (4) Gel electrophoresis for PCR product

1N*- Normal reaction as seen in the figure there is no band* ***1M****-Mutant reaction , the band is seen, which means that this is a homozygous mutant case* ***2N, 2M*** *represents a heterozygous mutant control. DNA-Ladder control DNA*

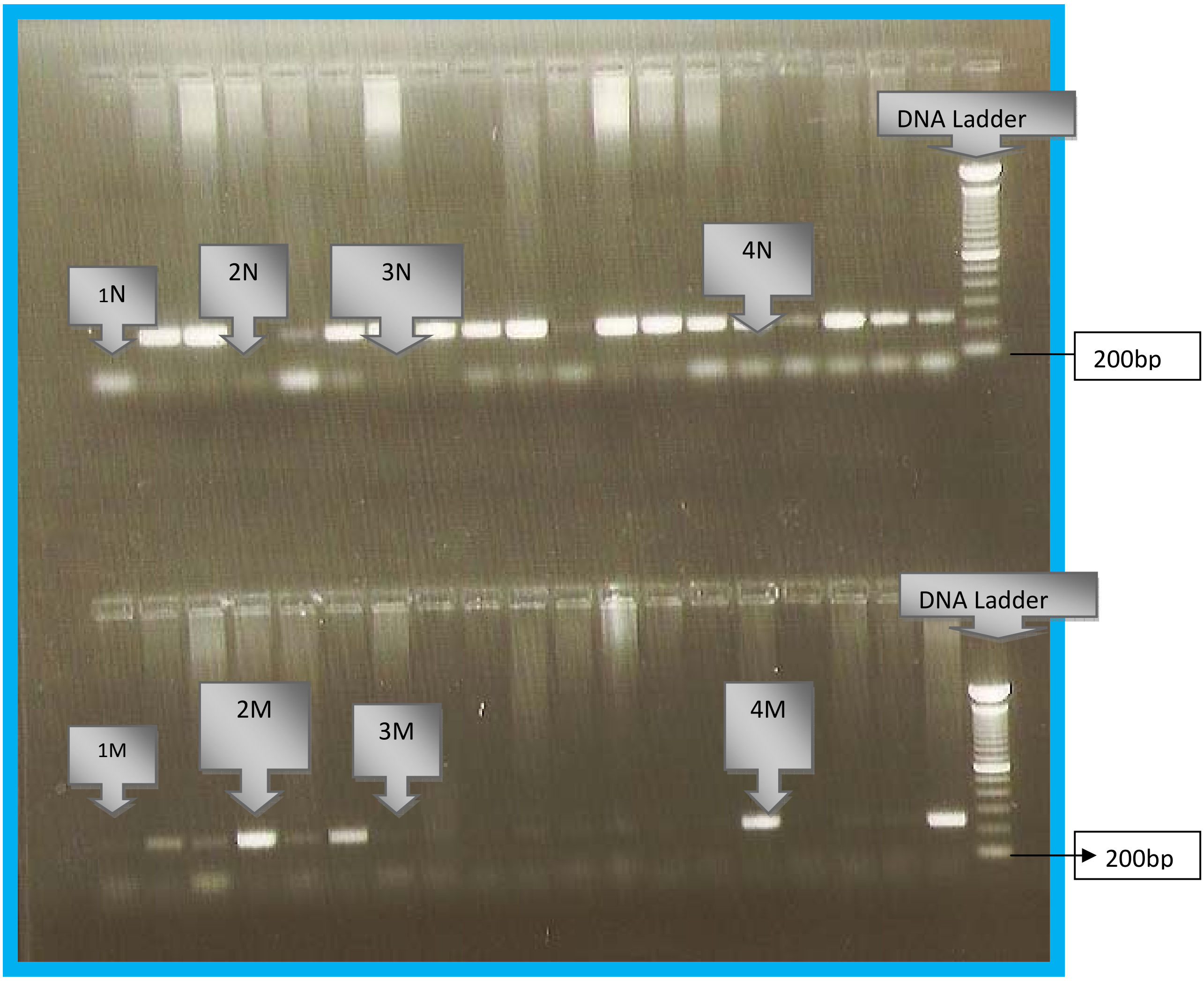


Fig (5) Gel electrophoresis for PCR products

From figure 5 we can see the different possible products of PCR, for example we can see the homozygous mutation (2M, 2N), we also can see the homozygous normal product (3N, 3M), the heterozygous mutant is also seen(4M, 4N), finaly we see the failed PCR (1N, 1M)

N-Normal primer reaction

M-Mutant primer reaction

Data Analysis

The data were analyzed using SPSS program version 15, P-value of < 0.05 was used for the significance of the results. Odds Ratio was used for detecting the power of the relationship between the determinant and the outcome. 95% confidence interval was calculated. Chi square was used to compare between the different independent groups.

Hypotheses

In addition to providing a frequency and descriptive analysis of the main background and independent variables, the study will provide cross-tabulation analysis to verify the level of statistical significance among different dependent and independent variables.

While it was possible to elaborate on high number of possible statistical relations due to the richness of the data, the present study focused on examining the relationship between factor V Leiden mutation and the poor pregnancy outcomes.

The following relationships represent the main areas of interest in the analysis and will be those consider in the results and discussion.

- The relationship between factor V Leiden mutation and recurrent pregnancy loss.
- The relationship between recurrent first trimester miscarriages and the Leiden mutation.
- The relationship between factor V Leiden mutation and recurrent pregnancy loss in the second trimester.
- The relationship between factor V Leiden mutation and IUFD.
- The relationship between factor V Leiden mutation and PIH.

Objectives

The main objective of our study was to explore the association between Factor V Leiden mutation and poor pregnancy outcomes, mainly recurrent miscarriages and PIH.

Chapter Three

Resuts

1-General characteristics

The results of our study showed the mean age of participants is 32 years, while the mean marriage age is 19 years. It is worth mentioning that about 10% of participants are smokers. *Figure 6* shows that refugees constitute 61% and 70% of cases and controls respectively. Most of them live in camps (69%), while the rest about (30%) reside either in city or village as shown in *figure (7)*. Evidently about 80% of participants not exceeded the low educational level and only 20% has some higher educational level as it is shown in figure *(8)*.

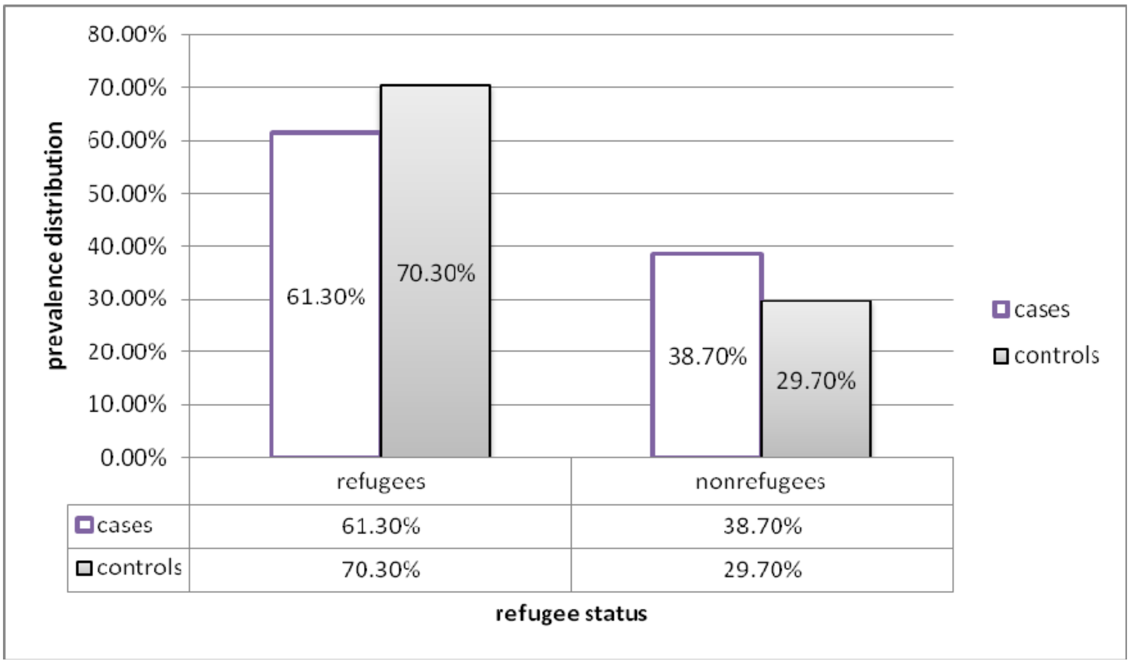


Fig (6) - Refugee status of participants

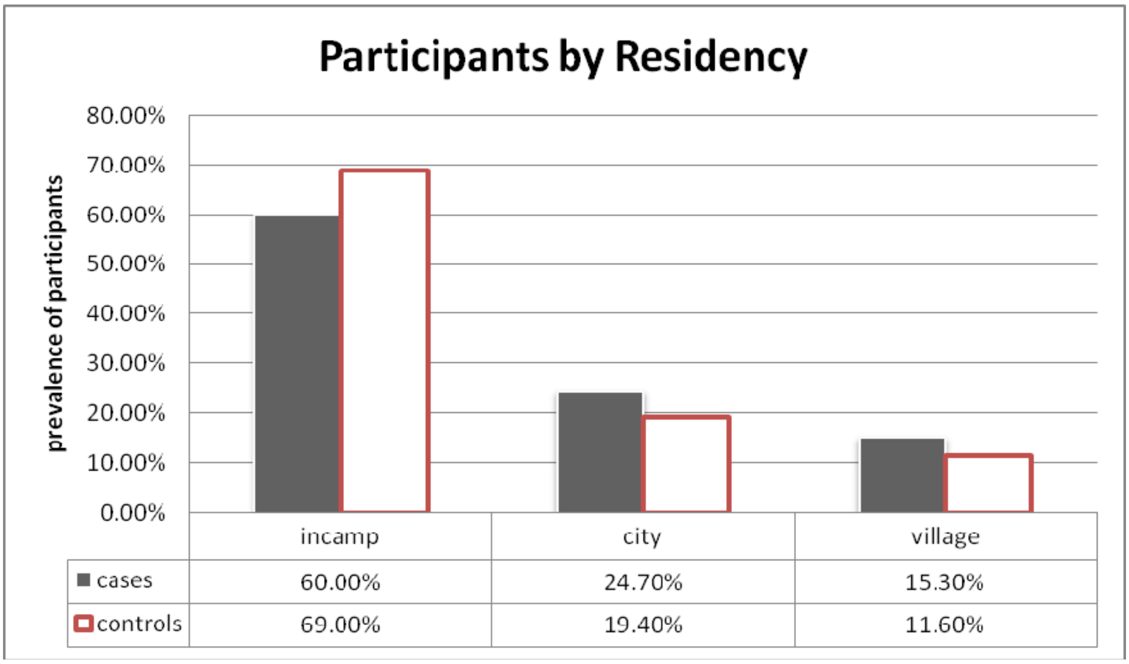


Fig (7)-Distribution of participants by residency

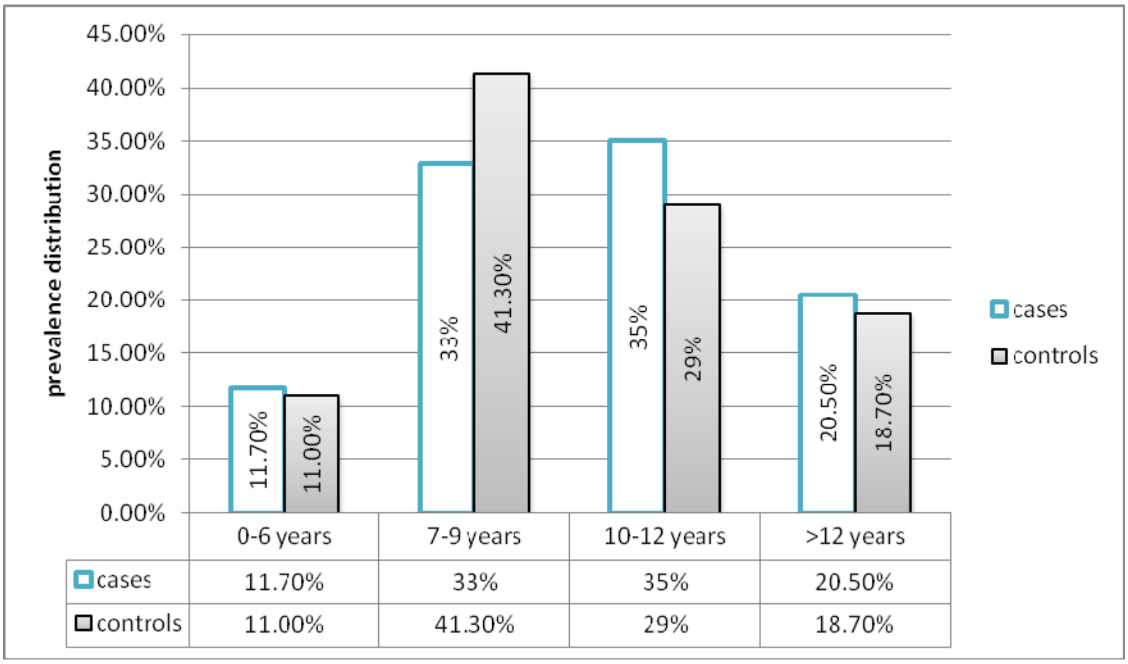


Fig (8)-Distribution of participants by educational level

Figure 9 demonstrates that more than 70% of our participants in both groups are overweight or obese with body mass index (BMI) between 25 and 40.

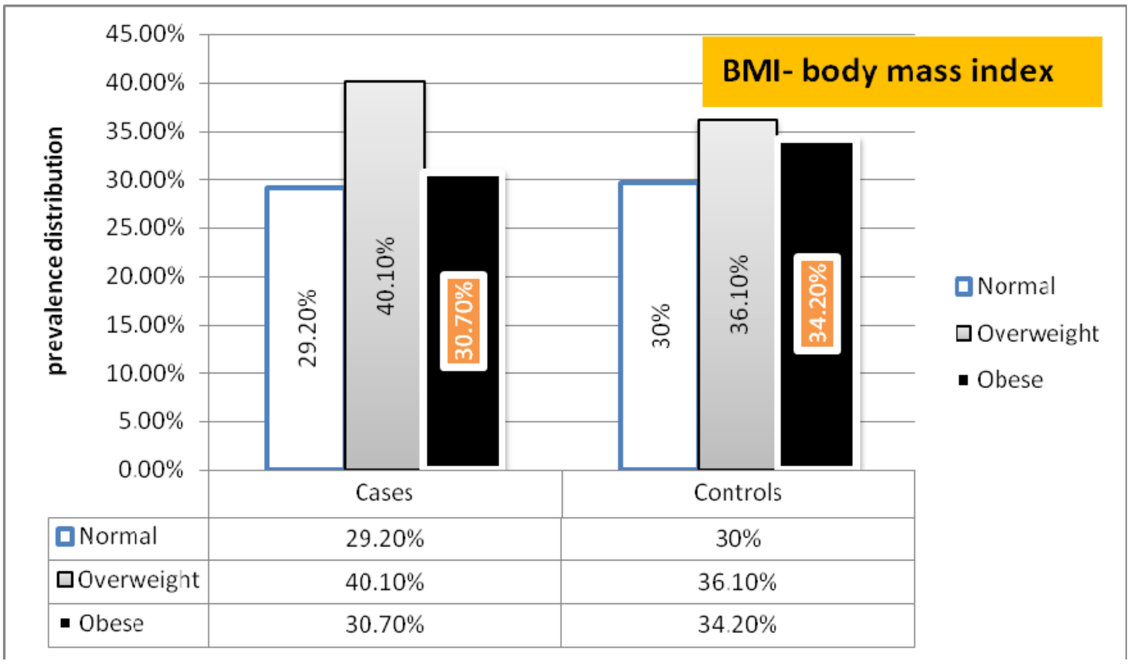


Fig (9)-Distribution of participants by BMI

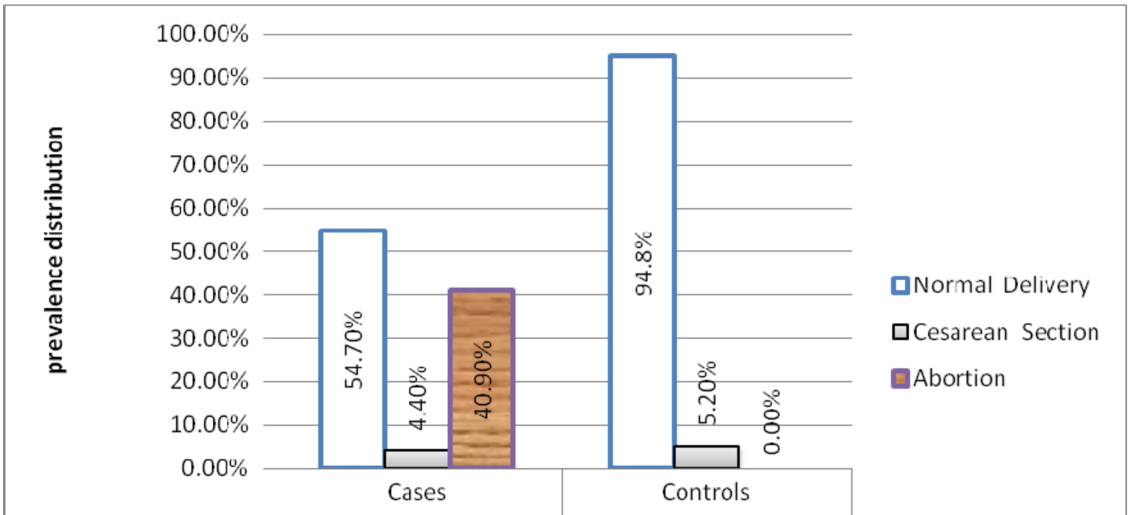
2-Obstetric History

2.1-Number of pregnancies, deliveries and abortions

The total number of pregnancies among cases was calculated to be 1009 pregnancies, 510 (50.5%) were successful, while 499 (49.5%) ended with miscarriages. However the total number of pregnancies among the control group was 725 pregnancies and all were successful.

2.2-First pregnancy outcome

The prevalence of different outcomes of the first pregnancy showed that 75 (54.7%) and 147 (94.8%) for cases and controls respectively ended with normal delivery, while only 6 (4.4%) of cases and 8 (5.2%) of controls ended with cesarean section *(Fig 10).* On the other hand 56 (40.9%) of first pregnancy among the case group ended with abortion (primary abortion) and 59.1% are with secondary abortions.



	Cases	Controls
Normal Delivery	54.70%	94.8%
Cesarean Section	4.40%	5.20%
Abortion	40.90%	0.00%

Fig (10) - First pregnancy outcomes

2.3-Types of poor pregnancy outcomes among the cases group

Poor pregnancy outcomes that are of primary interest are pregnancy induced hypertension which was detected in 20 of the cases (14.6%) and recurrent miscarriages that were seen in 117 (85.4%) of them (figure 11)

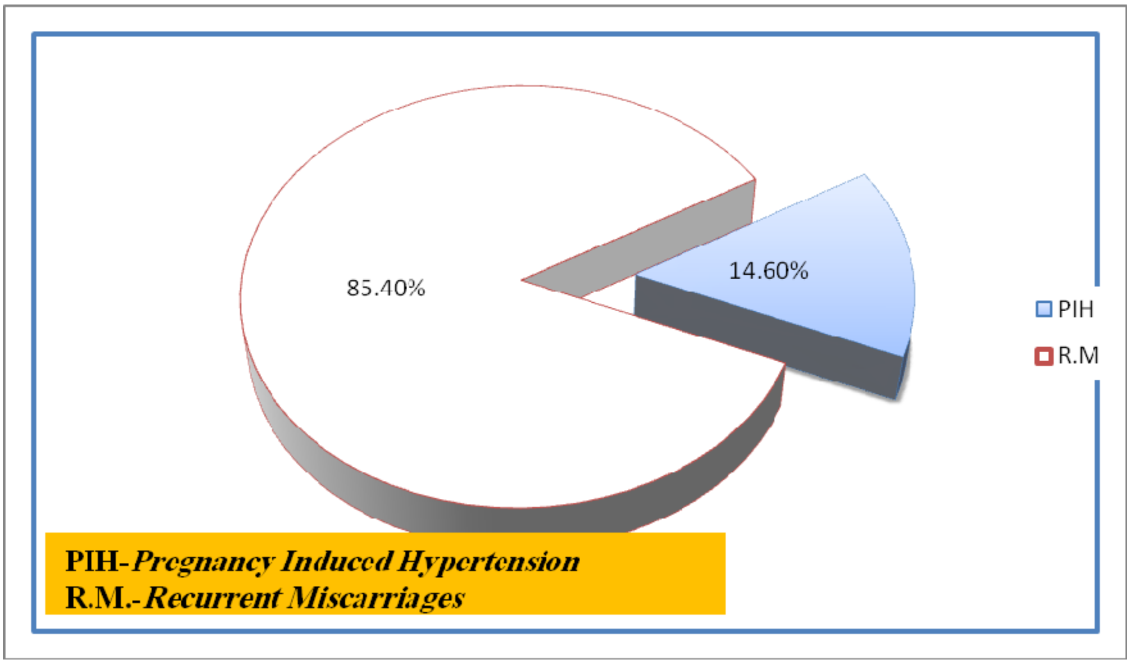


Fig (11)-Types of poor pregnancy outcomes

2.4 –Pregnancy Losses

In the current study recurrent miscarriages were classified into three categories: *first trimester abortions*, *second trimester abortions and Intra Uterine Fetal Death (IUFD). Figure (12)* clearly demonstrates that most

losses occurred within the first trimester, while the other two types show comparable frequencies.

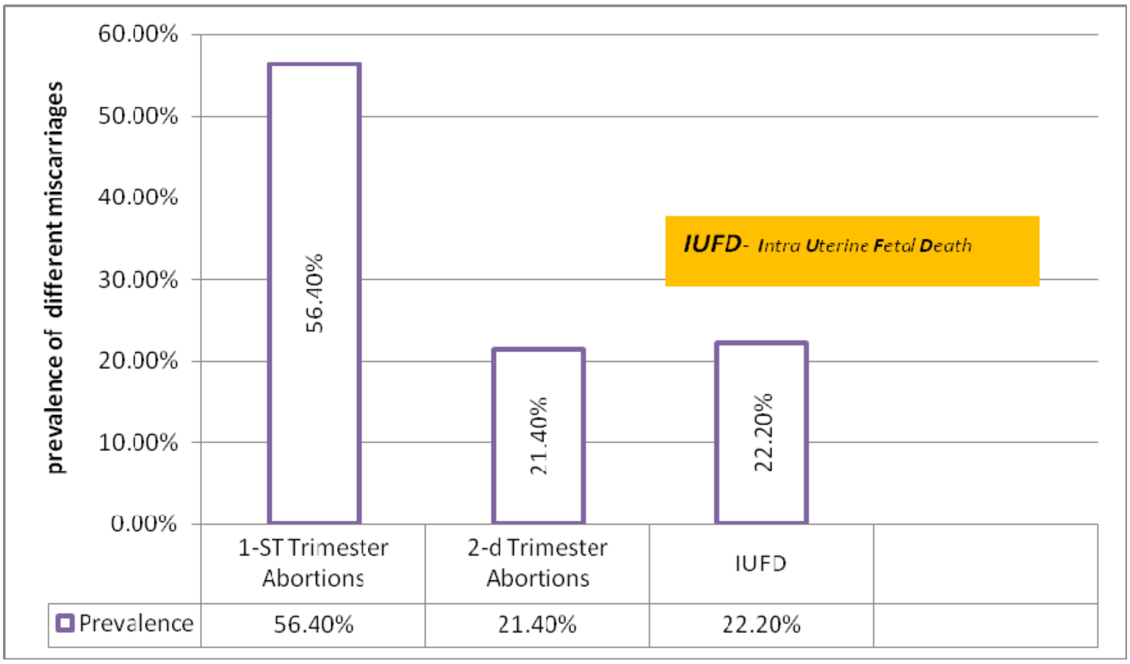


Fig (12) - Miscarriages types among cases

3-Family history of adverse events

Evidently medical and obstetrical family history constitute an important aspect in evaluating the health of individuals, therefore family history received special consideration when the questionnaire has been designed. Data analysis identified different adverse events that happened among the participants` families. These events could be classified into two major groups, namely *thromboembolic events* and *poor pregnancy outcomes*.

Figure 13 clearly demonstrates that the overall prevalence is significantly higher among cases group than that among the controls group.

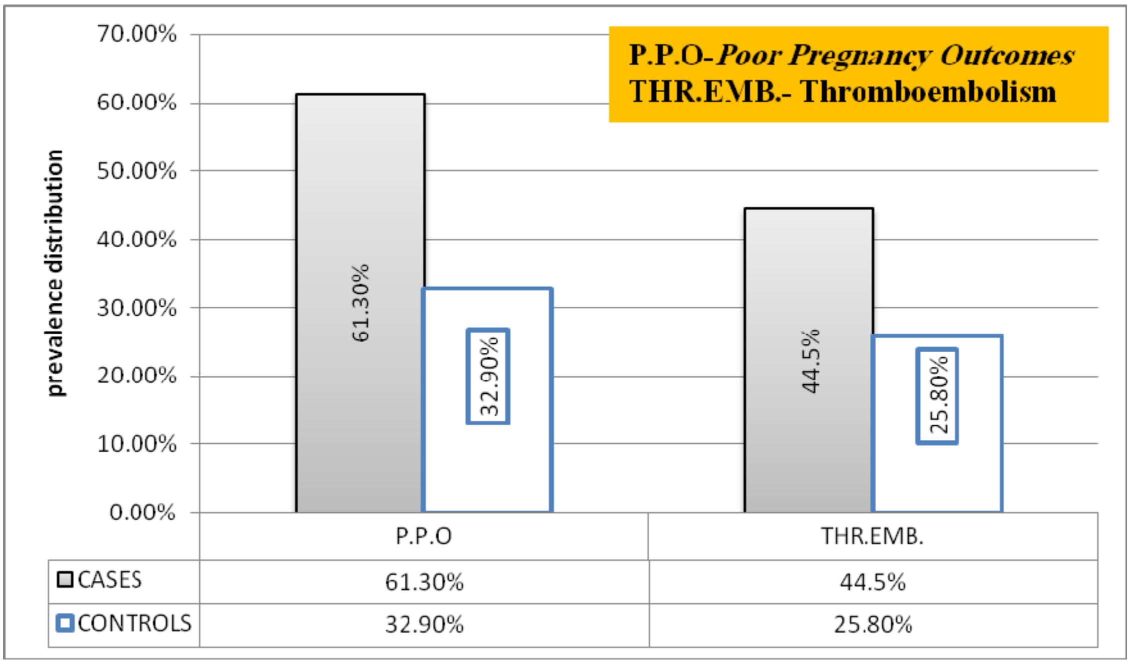


Fig (13) – Prevalence of adverse pregnancy outcomes among participants` families

3.1 Thromboembolic events

Table 1 describes the thromboembolic events that were revealed after analyzing the family history of our participants. Clearly, thromboembolic events among cases families are significantly higher than controls families. [P-0.001, Odds Ratio-2.308, 95% CI (1.410, 3.778)]

Table (1) Prevalence of thromboembolic events among participant's families.

	Cases(137)		**Controls(155)**	
	Frequency	**Prevalence**	**Frequency**	**Prevalence**
Ischemic Heart Disease	51	37.2%	35	22.6%
Pulmonary Embolism	15	10.9%	6	3.9%
Deep Vein Thrombosis	22	16.1%	6	3.9%
Overall	61	44.5%	40	25.8%

3.2-Prevalence of Poor Pregnancy Outcomes among Participant`s Families

Different types of poor pregnancy outcomes were analyzed namely family history of *recurrent miscarriages*, *IUFD, and preeclampsia*, as described in table 2. The difference between the two groups was significantly higher among cases compared to controls. [p=0.00, odds ratio =3.232, 95% CI= (2.000, 5.223)].

Table (2) Prevalence of poor pregnancy outcomes events among participant's families

	Cases (137)		Controls(155)	
	Frequency	Prevalence	Frequency	prevalence
Recurrent miscarriages	61	44.5%	40	25.8%
Pre-Eclampsia (PET)	15	10.9%	8	5.2%
Intra Uterine Fetal Death (IUFD)	28	20.4%	23	14.8%
Overall	84	61.3%	51	32.9%

.

4-Factor V Leiden mutation results

4.1- Prevalence of Factor V Leiden mutation among participants

Factor V Leiden mutation distribution showed higher prevalence among cases group than controls group. The mutation was detected in 35 out of 137 cases (25.5%) and in 13 out of 155 controls (8.4%) [P- Value =0.00, Odds Ratio=3.748, 95% CI (1.888, 7.439)].The results are shown in fig (14).

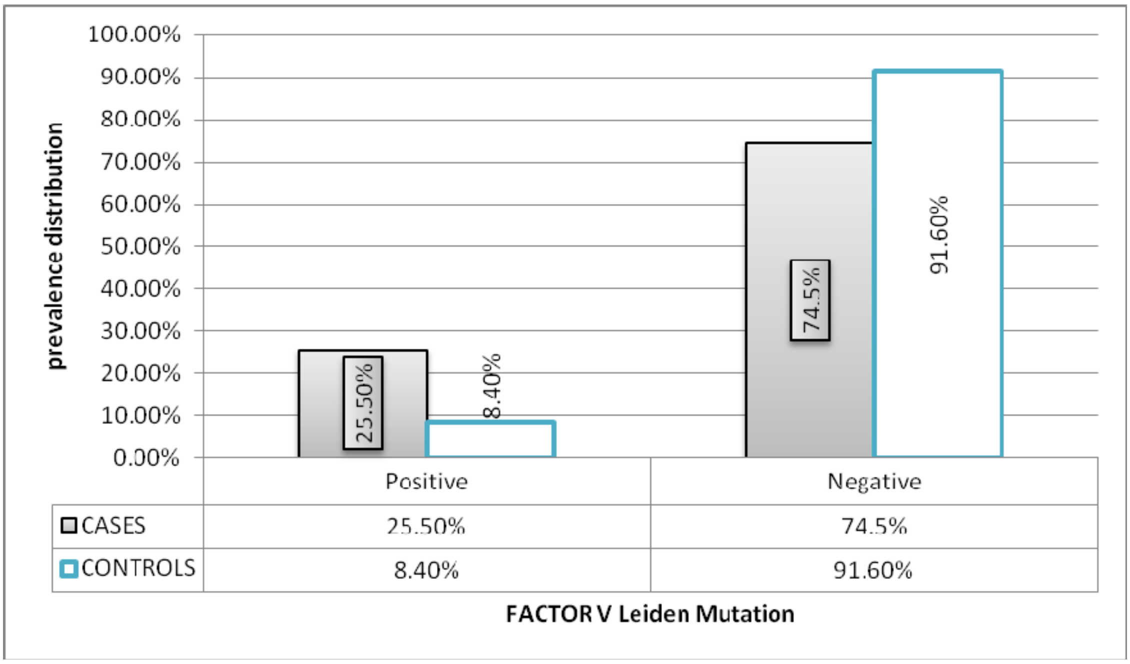


Fig (14) Leiden Mutation Prevalence among participants.

4.2-Genotypes

Figure 15 illustrates Factor V Leiden mutation among the cases groups includes 3 (8.6%) in the homozygous genotype and the rest 32 (91.4%) in the heterozygous genotype. No homozygous genotype were detected among the controls group

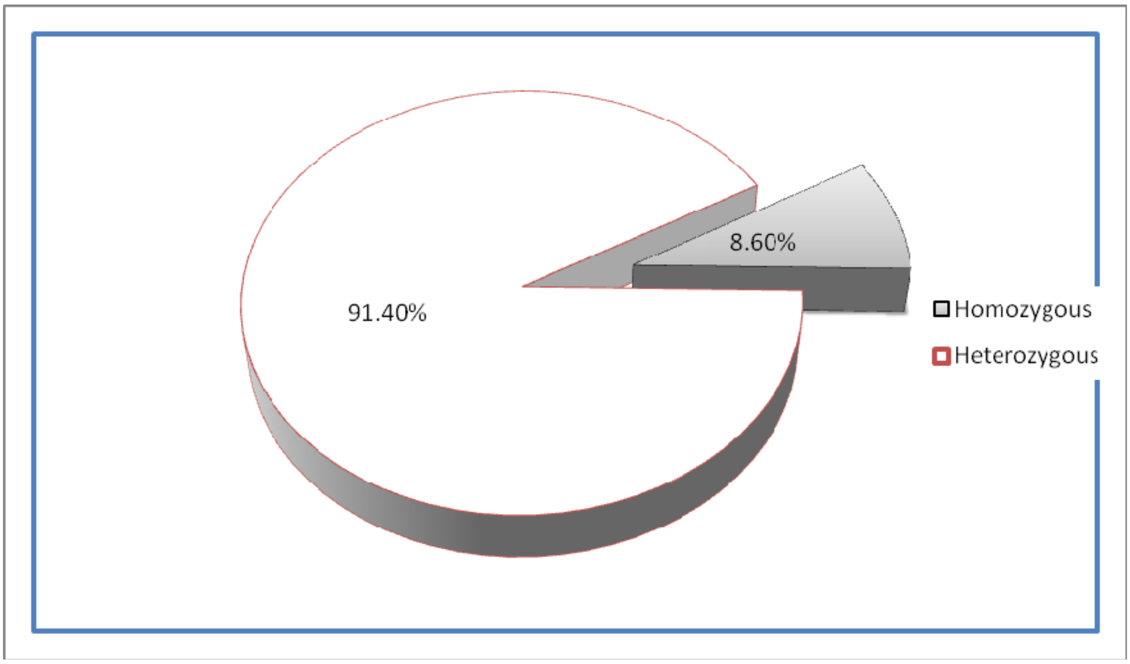


Fig (15) – Factor V Leiden mutation Genotypes

4.3-The relationship between Factor V Leiden mutation and different outcomes

In order to investigate the association significance between the Leiden mutation and poor outcomes among our participants, subjects were divided into four different categories:

- First trimester RM
- Second trimester RM
- IUFD
- PIH

Based on the results obtained from the analysis of PCR data, Factor V Leiden mutation was detected in 14 out of 66 women with first trimester abortion, (21.2%), a significant difference (p=0.012) is evident between the prevalence of the Leiden mutation in the test group compared to the control group (table 3). Leiden mutation was detected in 12 cases out of 25 women with second trimester abortion(48%), compared to 8.4% among the control group these results indicate a highly significant difference (p=0.00)for the prevalence of the mutation between the two groups, see table (3). Factor V Leiden mutation was also found significantly associated with IUFD (p=0.001) as shown in table (3). The prevalence of the mutation among the test group is 30.8% in comparison to 8.4% among the control group. In our study, we identified 20 cases with PIH. Factor V Leiden mutation was identified in only one of these cases which represents 5% prevalence among the test group, which indicates no significant difference between this group and the control group (p=1) as shown in table (3).

Table (3)- Comparison of the prevalence of Factor V Leiden mutation between women with different outcomes to the control group

Group	Total No.	Mutation carriers (%)	Comparison with the control group		
			P-value	Odds Ratio	95% CI
Control group	155	13 (8.4%)	-	-	-
Women with 1st trimester abortions	66	14 (21.2%)	.012	2.941	(1.296, 6.671)
Women with 2nd trimester abortions	25	12 (48%)	0.000	10.083	(3.828, 26.561)
Women with IUFD	26	8 (30.8%)	0.001	4.855	(1.772, 13.303)
Women with PIH	20	1 (5%)	1	0.575	(0.071, 4.646)

4.4 The relationship between the mutation in primary and secondary abortions

As mentioned above the cases group in our study was divided into two main subgroups based on whether they had primary or secondary abortion. Our data indicates that about 41% of the cases had primary abortions compared to 59% with secondary abortions. Factor V Leiden mutation was detected in 25% among women with primary abortion. A significant different was evident for the prevalence of Factor V Leiden mutation between the primary abortion group and the control group (p=0.004), as shown in table (4). Similar findings was also evident for the prevalence of the mutation between the secondary abortions group compared to the control group (p=0.00)

Table (.4) Comparison of the prevalence of Factor V Leiden mutation between women with primary and secondary abortions to the control group

Group	Total No.	Mutation carriers (%)	Comparison with the control group		
			P-value	Odds Ratio	95% CI
Control group	155	13 (8.4%)	-	-	-
Women with primary abortion	56	14 (21.2%)	.0004	3.641	(1.588, 8.348)
Women with secondary abortion	81	21 (48%)	0.000	3.823	(1.798, 8.131)

The difference of the prevalence of the mutation was not found to be significant when compared women with primary abortion to women with secondary abortion (p=1)

Chapter Four

Discussion & Recommendations

Discussion

Exploring the relation between Factor V Leiden mutation and recurrent miscarriages is a challenge. This is due to the fact that recurrent miscarriages are with multiple etiologies, where genetic factors are considered one of those etiologies. Advances in molecular genetic technology provide an accurate and reliable tool to precisely study the genetic abnormalities associated with many dise*ases*.

Abortion is considered a major problem in the world. Evidently the incidence of abortion is increasing in most developed and underdeveloped societies [6, 7]. The incidence of abortion is reported to be 4-8% among pregnant Palestinian women attending UNRWA antenatal care in West Bank [8]. There are several causes of abortion and 50% of abortions are with not established causes [6].

Thrombophilia, defined as the increase tendency of clot formation, is suggested to be one of the risk factors that contribute to the increasing incidence of poor pregnancy outcomes [6, 7]. There are two types of risk factors, namely acquired and inherited ones, which are associated with thrombophilia. Acquired factors include smoking, obesity, sedentary life,

pregnancy, oral contraceptive pills, immobilization especially in the postoperative period or after long pones fractures in addition to several other factors. However, inherited factors include deficiencies in protein S or C, mutations in some of the proteolytic cascade proteins, namely factor II, factor V and Methylenetetrahydrofolate reductase (MTHFR) [6, 7]. In this study, our work focused on investigating the association between inherited type of thrombophilias namely Factor V Leiden mutation and adverse outcomes of pregnancy.

The role of some thrombophilias in fetal losses has been well-studied in different populations. On the other hand the role of some inherited thrombophilias such as Factor V Leiden mutation and Factor II mutations are still under debate. The severity of the picture among Palestinians in the West Bank of Palestine is still poorly understood. Therefore, it is of great importance to explore the degree of the association between thrombophilias and poor pregnancy outcomes as the first step to provide the necessary clinical care that can minimize the chance of fetal loss during this stage. Based on this strategy a case control study was designed to analyze the relationship between Factor V Leiden mutation and some poor pregnancy

outcomes namely, recurrent miscarriages and pregnancy induced hypertension.

The results of our investigations show that about half of our study cases have ended with miscarriages while the entire control group had successful pregnancies. About 49% of pregnant women among the cases group had primery abortions. However, 14% of these cases had pregnancy induced hypertension and about 86% of them had recurrent miscarriages. Recurrent miscarriages have been mostly identified in the first trimester of pregnancy among the cases group, while the second trimester abortions and IUFD cases were with comparable frequencies.

Family history received special consideration in this study since it constitutes an important aspect in evaluating the health of individuals. This study clearly demonstrated the presence of statically difference of the adverse events among participant's families. For example, thromboembolic event and poor pregnancy outcomes are two events that have been determined. The distribution of those events was clearly apparent among the study group in comparison to the controls group. It became evident that recurrent miscarriages, intra-uterine fetal death and pre-eclampsia are

significantly higher among the case study group in comparison to the controls group.

The prevalence of Factor V Leiden mutation was tested and calculated in both case and control groups. The presence of Factor V Leiden mutation was predominately higher among cases group compared to the controls group. The prevalence of the mutation among cases group was about 25% while it was found to be about 8% among controls group. This result clearly demonstrates the association between poor pregnancy outcomes among a significant fraction of the case study group and Factor V Leiden mutation.

Factor V Leiden mutation, involved in the etiology of poor pregnancy outcomes, and has been proposed as one of the leading factors that is associated with poor pregnancy outcomes. For example, Kupferminc et al. [15] showed that pregnant women with serious complications have higher incidence of Factor V Leiden mutation predisposing them to higher risk of developing thrombosis compared to the control group. Similarly, Finan et al [16] reported that Lebanese women with recurrent idiopathic abortions had a significant difference of the prevalence of Factor V Leiden mutation in favor of the cases. Moreover, a study on Tunisian women showed clearly the positive association between the presence of Factor V Leiden mutation and

recurrent abortions [17]. In Italy, Martinelli et al [18] also demonstrated the association between unexplained late fetal loss and Factor V Leiden mutation. Similar conclusion has been reached between the Factor V Leiden mutation and recurrent miscarriages among Greek women [19] and among Italian women [20]. It has been documented that the prevalence of the mutation was significantly higher in women with pre-eclampsia than in women with normal pregnancy [20]. Moreover the association of Factor V mutation with recurrent abortion was also reported among Israeli women [22, 24]. In another study Souza et al [25] showed that there was a strong association between Factor V Leiden mutation and fetal losses among Brazilian women. The overall finding of the association of Factor V Leiden mutation in women with pregnancy complications, compared to women without complications strongly suggest an important role of the genetic thrombophilias in the occurrence of recurrent miscarriages in the Palestinian society similar to several other societies.

On the contrary other studies did not find a significant association between Factor V Leiden mutation and thrombophilias. Preston et al. [26] failed to show such association between Factor V Leiden mutation and recurrent fetal losses among European women participating in the European

prospective cohort study on thrombophilia. Similarly, Dizon-Townson et al. [27] reported similar findings in their study involving 40 American women having recurrent fetal losses.

This discrepancy can be explained based on the genetic background variations in different populations. In addition, the difference in sample size number between the various studies may be a good determinant in observed opposite conclusions. The, Dizon-Townson et al. [27] study included only 40 women while the present study and other previous studies used a much larger sample size.

In our study most women about 92% with RM who possess the Factor V mutation have heterozygous genotype, while only about 8% have homozygous genotype, while all control subjects with the mutation have heterozygous genotype.

Our result shows that although miscarriages are significantly higher among women in the first trimester (as expected) compared to those in the second trimester , however the association between Factor V Leiden mutation with recurrent miscarriages is much more significant with the second trimester abortions (48% second trimester vs. 14% first trimester) .

The current data also indicates a significant association between the presence of Factor V Leiden mutation and IUFD. However, no association could be established between the mutation and PIH. These results are in agreement with those reported by Martinelli al [[18]] on the association between Factor V Leiden mutation and fetal losses. Interestingly, our finding for the prevalence of the mutation in the control group (8.4%) is similar to that reported for the Israeli Arab population (8.5%); no data is currently available on the prevalence of the Factor V Leiden mutation among our population.

The following two tables (table 5 & 6) provide a summary of several previous reports that are in agreement (like our study) or disagree with the role of Factor V Leiden mutation in the development of adverse effects on pregnancies outcome.

Table. (5) Different Studies that support the association between factor V Leiden mutation and recurrent miscarriages

Author	**Target population**	**Prevalence of Factor V Leiden mutation**		**P-value**	**Odds ratio 95% Confidence interval**
		Cases (frequency)	Controls (frequency)		
Younes [22]	Israeli	19% (15/78)	6% (8/139)	<.05	3.17(1.03, 9.8)
Brenner [24]	Israeli	32% (24/76)	10% (11/106)	<.001	4(1.8, 8.8)
Foka [19]	Greek	19% (15/80)	4% (4/100)	.003	5.5(1.7, 17)
Grandone [20]	Italian	16.3% (7/43)	4.2% (5/118)	.01	4.39(1.3, 14.7)
Ridker [28]	American	8% (9/113)	3.7% (16/437)	.05	2.3(1.0, 5.2)
Souza [25]	Brazilian	7.1% (4/56)	1.6% (6384)	---	4.9(1.3, 17.8)

Table. (6) Different Studies that failed to show the association between factor V Leiden mutation and recurrent miscarriages

Authors	Target population	Prevalence of Factor V Leiden mutation		P-value	Odds ratio 95% Confidence interval
		Cases % (frequency)	Controls % (frequency)		
Preston[26]	European	26.9% (38/141)	23.5% (93/395)	.04	0.9 (.5-1.5)
Dizon-Townson[27]	American	0.0% (0/40)	0.0% (0/25)	….(*)	….(*)
Balasch[23]	Spanish	1.8 (1/55)	2.0% (1/50)	….(*)	(0.5-5.5)
Hashimoto[21]	Japanese	0.0% (0/52)	0.0 % (0/55)	….(*)	….(*)

(*) - *The values were not calculated in the concerned references.*

What are the clinical implications of these results? Anticoagulant therapy is apparently effective in reducing the incidence of adverse pregnancy outcomes in women with another thrombophilic condition, the antiphospholipid-antibody syndrome in Martinelli et al, [18] This therapy could also favorably improve the outcome of pregnancy in women with thrombophilic mutations who previously had pregnancy with fetal loss. However, before considering anticoagulant therapy during subsequent

pregnancies in these women, we need to know whether the presence of Factor V or prothrombin mutations also predispose women with recurrent unsuccessful pregnancies.

In conclusion, the prevalence of Factor V Leiden mutation is three times higher among cases group (25.5%) compared to controls group (8.4%) Therefore, screening for the presence of FV Leiden mutation is highly recommended. However, further studies on the presence of other mutations in the prothrombin or in the MTHFR genes are recommended to have a broad comprehensive understanding of the effect of thrombophilias on poor pregnancy outcomes

Recommendations

The result of our case-control study clearly shows significant correlation of factor V Leiden mutation in women with poor pregnancy outcomes compared to women with normal pregnancy. It is also evident from our study that abortion represents a major health problem in our society. However it is underestimated due to the high fertility rate among the population. The obtained data emphasize the importance of this study in identifying one of the risk factors that play a significant role in abortion. However, in our society, knowledge about the spectrum of other factors that lead to abortion is largely unknown. Thus based on our findings, we recommend the following:

1. There is a desperate need for more elaborate studies on thrombophilias and the role they play in poor pregnancy outcomes such as recurrent miscarriages, preeclampsia and abruption placenta.
2. Detailed studies are also needed on the relationship between different types of thrombophilias and thromboembolic events among Palestinians.
3. Conduct studies especially designed to reveal the prevalence of the different types of thrombophilias within our population including

mutations in the genes coding factor II and methylentetrahydrofolate reductase and their impact on poor pregnancy outcomes.

4. Increase the awareness of the medical personnel with regard to thrombophilias and its role in different thromboembolic events among our population.
5. Increase the awareness of the population about the risk factors that may increase the risk of developing thromboembolic events if are associated with thrombophilias.
6. Conduct studies about the possible prophylactic management of thrombophilias in risky people especially during pregnancy.
7. Explore the possibility of a screening policy for people who are at risk for thrombophilias.

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جامعة النجاح الوطنية

كلية الدراسات العليا

علاقة الطفرةالوراثية " معامل لايدن" بالنتائج السلبية للحمل

إعداد

خالد عبد القادر إسماعيل شلباية

إشراف

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قدِّمَت هذه الأطروحة إستكمالاً لمتطلبات درجة الماجستير في الصحة العامة بكلية الدراسات العليا في جامعة النجاح الوطنية، نابلس، فلسطين.

2007

علاقة الطفرةالوراثية " مُعامِل لايدِن" بالنتائج السلبية للحمل

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إشراف

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ملخص

الحمل عبارة عن حالة فيسيولوجية في حياة المرأة إلا أن الحمل قد ينتهي سلبيا، مثل الإجهاض المتكرر، أو قد ترافقه أمراض مثل ارتفاع ضغط الدم خلال فترة الحمل.

في عام 1993 تم إكتشاف طفرة وراثية في عامل التخثر الخامس (طفرة لايدن)، وقد أظهرت الدراسات أن لهذه الطفرة الوراثية دور هام في حدوث العديد من النهايات السلبية للحمل في بعض المجتمعات. مثل الإجهاضات المتكررة، إنفصال المشيمة المُبَكِّر، تَسَمُّم الحَمل، تأَخُّر نُمُو الجنين في الرَّحم.

بناء على ذلك فقد قمنا بعمل دراسة حول

علاقة *طفرة لايدن* بالإجهاضات المتكرِّرَة و إرتفاع ضغط الدم خلال الحمل في المجتمع الفلسطيني في مختبر الجينات بجامعة النجاح الوطنية. وقد اشتملت العينة الدراسية على 137 حالة مرضية تم إختيارها من مراجعات عيادات الوكالة في شمال الضفة الغربية أو حالات تم تحويلها من أخصائيي النسائية و الولادة في مدينة نابلس، و كذلك تم اختيار 155 إمراة من شمال الضفة الغربية اللواتي كن يراجعن مراكز وكالة الغوث في فترة الدراسة حيث مثَّلت هذه النِسوة المجموعة الضابطة.و بعد أخذ الموافقة الخطية من كل مشاركةعلى حِدَة تم سحب عينة دم لكل منهن

و عزل الحمض النووي اللذي استخدم في إستكشاف *طفرة لايدن* لكل حالة بإستخدام تقنية ال polymerase chain reaction.

و قد أشارت النتائج الى وجود علاقة ذات دلالة إحصائية ما بين ***طفرة لايدن*** و الإجهاضات المتكررة. فقد وجدت الطفرة عند 35 حالة من المجموعة التجريبية (25.5%) و عند 13 إمرأة من المجموعة الضابطة(8,4%) ***(الدالة الإحصائية=0,000)*** .هذا و قد تم تقسيم المجموعة التجريبية الى أربع مجموعات فرعية، حيث تكونت المجموعة الفرعية الأولى من نساء عانين الإجهاضات المتكررة في الثلث الأول من الحمل(66 إمرأة) ، أما المجموعة الفرعية الثانية فقد تكونت من نساء عانين الإجهاض المتكرر في الثلث الثاني من الحمل(25 حالة) بينما المجموعة الفرعية الثالثة إشتملت على الحالات التي عانت من إجهاضات الثلث الأخير من الحمل(26 حالة)، في حين أن المجموعة الفرعية الرابعة تكونت من نساءٍ عانين من ضغط الحمل المرتفع(20 حالة). وبعد تحليل النتائج تبين أن ***طفرة لايدن*** كانت متواجدة عند المجموعات الفرعية الأربعة بالنسب التالية مرتبة من الاولى حتى الرابعة (21.2%، 48%، 30.8%، 5%). و بعد التحليل الإحصائي للعلاقة بين وجود ***طفرة لايدن*** عند المجموعات سابقة الذكر و المجموعة الضابطة بإستخدام مربع كاي تبين وجود فروق ذات دلالة إحصائية هامة ما بين المجموعات الفرعية الثلاث الأولى (كلٌ على حدة) و المجموعة الضابطة حيث كانت الدالة الإحصائية بالتتالي(0.012 0.000 0.001) ، ولكن مثل هذه الفروق ذات الدلالة الإحصائية لم نجدها في دراستنا عند مقارنة وجود ***طفرة لايدن*** ما بين المجموعة الفرعية الرابعة و المجموعة الضابطة ، حيث كانت الدالة الإحصائية =1

ومن النتائج المذكورة أعلاه يمكننا استنتاج عن وجود علاقة ذات دلالة إحصائيةما بين ***طفرة لايدن*** و بعض النتائج السلبية للحمل عند النساء المشاركات في هذه الدراسة.

و عليه فإننا ، نوصي بعمل مسح للتعرف على نسبة إنتشار ***طفرة لايدن*** في المجتمع الفلسطيني.

و إجراء المزيد من الدراسات المشابه للدراسة الحالية حول هذه الطفرة ، و ذلك من أجل الخروج بنتائج داعمة لهذه الدراسة وذلك للإستفادة في التطبيقات السريرية في مثل هذه الحالات

دِراسَة حَولَ عِلاقَة الطَفرَة الوِراثية "مُعامِل لايدن "مع بعض النتائج السلبية للحمل

أثبت العديد من الدراسات بأن معامل لايدن منتشر بنسبة 8-14%بين سكان الدول العربية المجاورة و كذلك بين فلسطينيي الخط الأخضر،

وقد أثبت العديد من الدراسات على و جود علاقة ما بين هذا العامل الوراثي و العديد من أمراض الحمل مثل تسمم الحمل،إنفصال المشيمة الحاد،الولادة المبكرة،تأخر نُمُو الجنين و كذلك الإجهاضات المتكررة. .

هناك العديد من الدراسات العالمية التي تثبت إمكانية الوقاية من هذه الأمراض و ذلك بعد إكتشاف هذا العامل الوراثي عند الشخص المعني.

بناءً على ذلك و لأهمية الموضوع فقد قرر فريق من الباحثين في جامعة النجاح الوطنية نابلس-فلسطين القيام بدراسة مدى وجود هذا العامل الوراثي بين النساء اللواتي تعرضن لمثل هذه الأمراض ومقارنة ذلك مع نسبة وجوده عند نساء لم يتعرضن لمثل هذه الأمراض، و ذلك إستكمالا لمتطلبات درجة الماجستير في الصحة العامة.

وبناءً على ذلك وللأهمية العلمية و لمصلحة أبنائنا و بناتنا نرجو من حضرتكم الإجابة على هذا الإستبيان بدقةٍ متناهية.علماً أنّ المعلومات الواردة فيه ستُعامل بسريّةٍ تامة و لن ُتستخدم الاّ للبحث العلمي.

مع الإحتفاظ بِحَقِكُم الكامل بالإنسحاب من الدراسة في الوقت الذي تُقرّرون.

ح

لمزيد من الإستفسار الرجاء عدم التردد بالأتصال مع :

د.خالد شلباية

جوال: 0599674130

شاكرين لَكُم حسن تعاونكم.

علاقة الطفرة الوراثية(مُعامِل لايدن) ببعض النتائج السلبية للحمل

المركز الصحي: ☐ **رقم الملف :** ☐ **الرقم المتسلسل :** ☐

تاريخ الميلاد : ☐ **العمر عند الزواج :** ☐

الوزن: ☐ **الطول :** ☐

هل أنت لاجئة؟

☐ **نعم** ☐ **لا**

مكان السكن الحالي:

☐ **مدينة** ☐ **قرية** ☐ **مخيم**

زواج أقارب درجة ثانية: إبن (عم ،عمة،خال،خالة)

☐ **نعم** ☐ **لا**

درجة التعليم :

☐ **ابتدائي** ☐ **اعدادي** ☐ **ثانوي** ☐ **أعلى من ذلك**

المهنة :

☐ **ربة بيت** ☐ **موظفة** ☐ **أعمال خاصة**

العمر عند الحمل الأول : ☐ **العمر عند الولادة الأولى :** ☐

نتيجة الحمل الأول :

☐ **ولادة طبيعية** ☐ **ولادة قيصرية** ☐ **اجهاض**

د

عدد الحمول : ☐ عدد الحمول المكتملة : ☐ عدد حالات عدم اكتمال الحمل : ☐

عدد حالات عدم اكتمال الحمل في الثلث الأول : ☐

عدد حالات عدم اكتمال الحمل في الثلث الثاني : ☐

عدد حالات عدم اكتمال الحمل في الثلث الأخير ☐

هل يعاني أحد من اقربائك من أي من الأمراض التالية؟

#	السؤال	نعم	لا	لا أعرف
1	تجلطات قلبية			
2	تجلطات رئوية			
3	تجلطات وريدية			
4	هل عانت والدتك او شقيقة لك من ولادة مبكرة؟			
5	هل أنجبت والدتك او شقيقة لك أطفال دون 2500 غم؟			
6	هل عانت والدتك أو شقيقة لك من اجهاضات متكررة؟			
7	هل عانت والدتك أو شقيقة لك من تسمم حمل ؟			
8	هل عانت والدتك أو شقيقة لك من انفصال المشيمة الحاد؟			
9	هل عانت والدتك أو شقيقة لك من وفاة الجنين قبل الولادة ؟			
10	هل عانت والدتك أو شقيقة لك من وفاة الجنين أثناءالولادة ؟			

ذ

ضعي اشارة (×) في المكان المناسب :

#	السؤال	نعم	لا	لا أعرف
1	هل تعانين من السكري؟			
2	هل عانيتِ من مرض سكري الحمل سابقا؟			
3	هل تعانين من ارتفاع ضغط الدم؟			
4	هل عانيتِ من تسمم الحمل؟			
5	هل عانيتِ من إرتفاع ضغط الدم خلال الحمل؟			
6	هل تعانين من دوالي الساقين؟			
7	هل عانيتِ من تجلطات سابقة في احدى ساقيك؟			
8	هل استخدمتِ حبوب منع الحمل ؟			
9	هل أصبت بجلطة في الساق أثناء إستخدام حبوب منع الحمل ؟			
10	هل انتِ مدخنة ؟			
11	هل سبق وأن ولدتِ ولادة مبكرة ؟			
12	هل سبق وأن ولدتِ أطفال دون وزن 2500غرام؟			
13	هل سبق وأن عانيت من وفاة الجنين قبل أو أثناء الولادة ؟			
14	هل سبق وأن عانيت من إنفصال حاد في المشيمة؟			

وَ شُكْراً لِحُسْنِ التَّعاوُنْ